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THE HEAT RESISTANCE OF THE SPORES OF B. BOTULINUS AND ALLIED ANAEROBES. XI

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As a part of a broad study dealing with the bacteriology and biochemistry of B. botulinus, the heat resistance of the spores of this and allied anaerobes has been investigated. A brief analysis of the accumulated data made on 1804 spore suspensions and 65 soil specimens naturally or artificially contaminated with the spores of B. botulinus, has revealed a number of important scientific and practical facts. As the detailed preparation and publication of the numerous experiments embracing over 42,807 cultures may be delayed for several months, it is deemed advisable to report the main tentative conclusions. The technic used in these studies is in principle identical with that employed by Bigelow and Esty[1] in their work on the heat resistance of thermophilic bacteria. In the course of the investigation minor modifications in the preparation of the cultures and the subcultures of the heated spore suspensions have been adopted in order to eliminate the danger of laboratory contaminations. Extensive experience has definitely proved the broad applicability and absolute reliability of this standard method.

COMPARATIVE RESISTANCE OF SPORULATING ANAEROBES TO MOIST HEAT

(*a*) *B. botulinus.*—A comparative study of 109 strains of B. botulinus, which originated from numerous field specimens, soils and vegetables as well as food products responsible for 26 human and 9 chicken outbreaks of botulism, reveals two facts: (1) certain strains sporulate irregularly and poorly, and (2) the heat resistance fluctuates within wide limits. The spores employed in these tests have been produced in canned pea infusion—peptic-digest broth (equal parts P_H 8.4). After an incubation of 10 days at 35 C. the culture has been concentrated by centrifugalization and the sediment suspended in 50 c c of a M/15 phosphate solution of a P_H of 7.00 to 7.12 (Sorensen mixture of Na_2HPO_4 and KH_2PO_4). The mixture has been vigorously shaken in order to produce a uniform suspension, and 2 c c amounts have been delivered with the aid of a sterile buret into tubes of hard glass with a uniform bore, thickness, etc. These tubes have been sealed in an oxygen blast and heated at 105 C. (221 F.) in a DeKhotinsky electric oil bath. The temperature has been controlled by means of a thermo-regulator and kept uniformly distributed throughout

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[1] Jour. Infect. Dis., 1920, 27, p. 602.

the bath by a turbine stirrer device. Subcultures of the heated spore suspensions have been made in a 0.5% glucose-beef-heart-peptic-digest broth (PH 7.0-7.4) stratified with vaseline and incubated at 35 to 37 C.

The 109 strains are divided into 3 groups according to their toxicologic and serologic characteristics: type A, 78; type B, 30; nontoxic, 1 strain. Their relative heat resistance is shown in table 1.

At a temperature of 105 C. (221 F.), the average survival time of the spores of 78 strains of B. botulinus, type A, is 40.1 minutes, while the minimum is less than 9 minutes and the maximum 85 minutes. The survival time of the spores of 30 type B strains at the same temperature is on the average 23.7 minutes, with a minimum of less than 6 minutes and a maximum of 60 minutes. The average resistance of the 109 strains of B. botulinus is 35.2 minutes at 105 C. (221 F.). Spores of type B are apparently less heat resistant than those of type A strains. It is, however, evident that under exceptional circumstances strains and spores of a resistance equal to that recorded for type A strains may be encountered in nature or may be produced artificially in the laboratory.

TABLE 1

RELATIVE HEAT RESISTANCE OF THREE GROUPS OF B. BOTULINUS STRAINS

Resistance at 105 C. (221 F.) in Minutes	Number of Type A Strains	Number of Spores Heated	Number of Type B Strains	Number of Spores Heated	Total Number of Type A and B Strains
Less than 12......	3	1 no count 0 2 = 10 M.[*] and 100 M.	10	1 no count 0 100 M. to 1 B.	13
12 to 21...........	9	250 M. to 1,300 B.	5	200 M. to 3 B.	14
21 to 42...........	33[†]	25 M. to 3 B.	7	130 M. to 1,200 M.	40
42 to 60...........	16	130 M. to 3 B.	4	100 M. to 200 M.	20
60 to 90...........	18	250 M. to 12 B.	4	75 M. to 535 M.	22
Less than 12 to 90	79[†]	10 M. to 12 B.	30	75 M. to 3 B.	109

[*] M. = million; B. = billion.

[†] The spores of the nontoxic strain survived 30 minutes but were killed in 33 minutes. Two strains (1 A and 1 B) failed to produce spores either in pea peptic digest broth or brain medium; 9 strains (4 A and 5 B) were grown in minced sheep brain medium with a natural reaction after it was noted that pea peptic digest broth failed to yield a sufficient number of spores to make comparative tests. Spores of 4 strains cultivated and tested in this manner were destroyed in less than 12 minutes.

(*b*) *B. sporogenes.*—The heat resistance of 33 strains of B. sporogenes has been determined at 100, 105 and 110 C. The spores have been produced either in pea peptic digest, veal infusion-peptic-digest-gelatine or brain medium. The following heat resistances have been recorded:

100 C. (212 F.) 10 to 150 minutes; average survival time 72.5 minutes.
105 C. (221 F.) 4 to 45 minutes; average survival time 12 minutes.
110 C. (230 F.) 1 to 12 minutes; average survival time 3.3 minutes.

The cultures of heated spore suspensions exhibit the property of retarded germination. In several instances it has been noted that cultures failed to grow until after 100 days of incubation at 35 C.

(*c*) *B. tetani.*—The spores of 24 strains of B. tetani purified and described by H. H. Heller,[2] produced either in pea peptic digest broth, brain medium, double strength veal infusion-peptic-digest-gelatine or casein broth, exhibited the following heat resistance:

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[2] Ibid., 1922, 31, p. 31.

100 C. (212 F.) 15 to 90 minutes; average survival time 25 minutes.
105 C. (221 F.) 3 to 25 minutes; average survival time 9.2 minutes.

The maximum resistance was obtained in neutral brain medium. Germination of the heated spores in an optimum medium was not markedly retarded. All surviving spore suspensions showed growth within 6 days.

(*d*) *B. bifermentans.*—Three of the 4 strains of B. bifermentans (isolated and described by I. C. Hall[3]) survived 30 minutes, but were destroyed in 45 minutes at 100 C. The spores of one strain exposed to a temperature of 105 C. (221 F.) survived 18 minutes, but did not resist 21 minutes.

(*e*) *B. centrosporogenes.*—Three of 4 strains of B. centrosporogenes (isolated and described by I. C. Hall[3]) were destroyed in less than 30 minutes at 100 C. The fourth strain resisted 30 minutes but not 45 minutes at the same temperature.

(*f*) *B. welchii.*—One strain of B. welchii isolated from a heated soil specimen produced spores in sheep brain medium, which survived 24 minutes but not 27 minutes at 105 C. The spores of another strain were killed in 10 minutes but survived 5 minutes at 100 C.

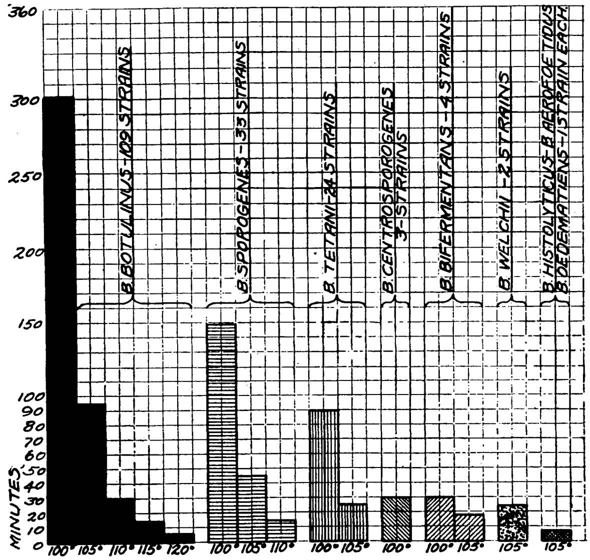

Chart 1.—Maximum heat resistance of sporulating anaerobes.

(*g*) *B. histolyticus, B. oedematiens and B. aerofoetidus.*—One strain each of B. histolyticus, B. oedematiens and B. aerofoetidus failed to survive 6 minutes at 105 C. The anaerobe isolated by Bengtson[4] from the larvae of Lucilia Caesar was destroyed in less than 15 minutes at 100 C. The subcultures of the anaerobes mentioned under *d, e, f* and *g* exhibited no retarded germination.

The maximum heat resistances of the various anaerobes tested at different temperatures are shown in chart 1. It is definitely demonstrated that B. botulinus produces spores of extraordinary resistance toward moist heat.

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[3] Ibid., 1922, 30, p. 445.

[4] Pub. Health Rept., 1922, 37, p. 164.

Furthermore, a comparison of these data with those published by von Hibler,[5] Becker[6] and others indicates that the resistance of the spores of American strains of B. botulinus surpasses that of the sporulating anaerobes thus far investigated.

THE MAXIMUM RESISTANCE OF B. BOTULINUS

The maximum resistance to moist heat of B. botulinus spores artificially produced under optimum conditions of temperature, food, oxygen requirements, etc., and heated in a phosphate solution prepared from a mixture of M/15 Na_2HPO_4 and KH_2PO_4 to give a P_H value of approximately 7 is as follows:

4 minutes at 120 C. (248 F.)
10 minutes at 115 C. (239 F.)
33 minutes at 110 C. (230 F.)
100 minutes at 105 C. (221 F.)
330 minutes at 100 C. (212 F.)

The data of similar tests secured with suspensions containing billions of spores produced by the most resistant strains have been used in the compilation of the "ideal destruction curve" shown in chart 2. This curve includes the maximum resistance observed on 1,804 spore suspensions and shows the negative results obtained at the temperatures stated.

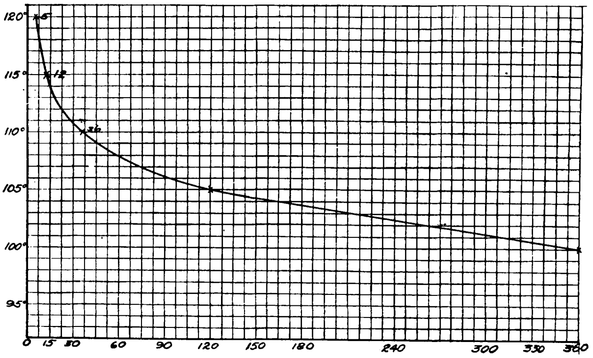

Chart 2.—Ideal destruction curve of B. botulinus strains 19, 23, and 97 grown in double strength veal peptic digest gelatine at 37 C. for 10 days, 60 billion in M/15 PO_4 solution, P_H.7.0.

VARIATIONS IN THE HEAT RESISTANCE OF SPORES

The heat resistance of the spores of B. botulinus varies considerably, depending on several factors, some of which are unknown. The influence of the composition and the reaction of the broths used for the productions of the spores have been studied on 24 different mediums. The following preparations have been tested; pea-juice-peptic digest broth, pea-juice gelatine (10%), pea-juice-peptic-digest gelatine (10, 15 and 20%), pea juice, spinach juice, spinach juice with 2% Berna peptone, spinach-juice-peptic digest, spinach-juice-peptic-digest gelatine (10%), double strength veal infusion with and without peptic digest and gelatine, peptic-digest broth with and without Berna peptone (1%), diluted pea juice, glucose-peptic digest with and without gelatine, brain natural reaction and brain with 2% peptone, asparagus juice with 1% Difco-peptone, alkaline egg medium, casein digest, beef-heart-peptic-digest broth and milk agar in anaerobic plates (surface growth). In a number of mediums the influence of the P_H, the salt and phosphate content has been investigated. It has been established that the production of abundant spores with a fairly consistent heat resistance is regular in a double strength veal infusion-peptic-digest (equal parts) 10% gelatine with a P_H of 7.4.

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[5] Untersuchungen über pathogene Anaeroben, 1908.

[6] Centralbl. f. Bakteriol., O.. I, 1920, 84, p. 71.

The spores which develop in certain mediums are always of very low resistance. Furthermore, it has been repeatedly noted that the spores generated in different flasks of the same medium, inoculated with equal amounts of the same stock culture and incubated for the same period, may show striking differences in heat resistance. These differences appear to be independent of the final reaction of the culture fluid within the range of P_H 6 to 8, the number and microchemical structure of the spores. Strains which sporulate poorly in liquid behave likewise in solid mediums.

TABLE 2

Results of Experiments on Heat Resistance of Spores

Flask	Final P_H	Millions of Spores Obtained and Heated	Resistance at 105 C. in Phosphate Mixture P_H 7.0		Retarded Germination
			Minutes +	Minutes —	
1	8.31	600	12	18	16 days
2	7.69	244	24	28	14 days
3	8.19	2,324	12	18	16 days
4	8.22	268	24	28	30 days
5	8.21	880	18	24	18 days
6	8.20	600	24	28	24 days
7	8.05	1,100	18	24	24 days
8	....	1,040	18	24	21 days

+ = survival time; — = destruction time.

One experiment is cited to illustrate this statement. Eight flasks of pea-peptic digest medium P_H 7.4 of the same lot were inoculated with strain 90 and each received 0.1 c c of the stock beef heart culture. The containers were incubated at 35 C for 10 days and the spores of each culture were heated separately in phosphate solutions of the same P_H prepared from the same standard mixture. The results are shown in table 2.

Supplementary tests have furthermore shown that striking variations are obtained when the spore suspensions are prepared and heated in the culture fluid in which they have been produced. For comparative tests it has, therefore, been found advisable to concentrate the spore material of several flasks containing from 800 to 1,500 c c of culture fluid by centrifugalization and to resuspend the sediment in a phosphate buffer solution of a neutral reaction. This phosphate mixture is easily duplicated, undergoes little or no change during the heating, and the spores tested in this solution have, as a rule, possessed the maximum resistance.

Several factors responsible for the peculiar variations in resistance deserve a more detailed discussion than is possible in this summary. They will be presented in the final report.

THE RESISTANCE OF B. BOTULINUS IN NATURAL AND ARTIFICIALLY INFECTED SOILS

For practical purposes it appeared advisable to determine the heat resistance of the spores of B. botulinus as found in nature. Suitable soil specimens selected from the collection of field samples which have been studied in connection with the problem on the distribution of this anaerobe in the United States have been used for this purpose. Definite amounts of 56 soil specimens originally collected in regions in which human or animal botulism had occurred and known to contain spores of B. botulinus have been weighed into large hard glass tubes containing 0.5% glucose-peptic digest-beefheart broth. These tubes have been sealed in an oxygen blast and heated in a bath of boiling water (99.5-100 C.) from 1 to 6 hours. The heated cultures have been incubated at 35 C. and observed daily. Growth has been judged by gas production, turbidity of the medium and the appearance of the meat. The presence of B. botulinus has been determined by toxin tests on mice and by deep agar shake cultures.

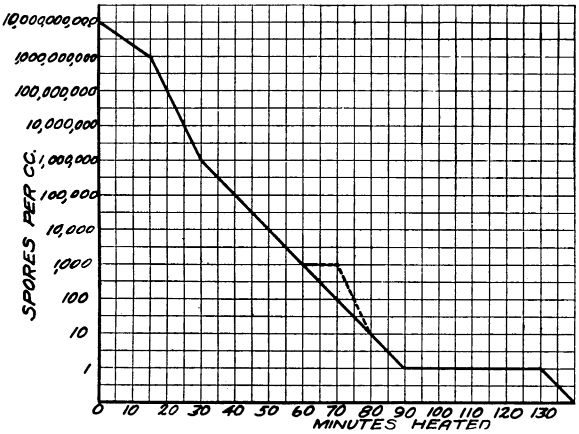

Chart 3.—Death rate of B. botulinus spores in phosphate M/15 KH_2PO_4 and NA_2HPO_4 at 100 C.

Three specimens of soil obtained from Colorado, New York and Washington contained a sufficient number of viable spores of B. botulinus to furnish toxic cultures after being heated. Two samples survived boiling for 1 hour and only 1 for 2 hours. All the samples heated for 150 minutes at 212 F. proved nontoxic and free from B. botulinus. These tests indicate that the average resistance of the spore of B. botulinus as found in approximately 20 gm. of naturally contaminated soil is less than 3 hours at 100 C. (212 F.).

Additional tests have been performed on 15 soil specimens, 6 sterile and 9 nonsterile, artificially inoculated with detoxified spores of B. botulinus and incubated for from 20 to 30 days aerobically or anaerobically at 27 C. It is known that B. botulinus developed in these soil cultures. Samples which contained sufficient toxin to kill guinea-pigs in from 20 hours to 5 days when fed, have been chosen for the heat resistance tests. Equal amounts of the soil suspension cultures have been treated in the manner stated. The data show that 8 of the 9 nonsterile soil samples developed toxic cultures after exposure to boiling water for 2 hours. All tubes heated for 150 minutes have been negative for B. botulinus. One sample of the 6 originally sterilized soil suspensions has become toxic after heating for 1 hour. The tubes heated for 2 hours have remained sterile. The spores of B. botulinus produced in sterile or nonsterile soil suspensions were destroyed in less than 3 hours. The second series of experiments fully confirm the results secured on naturally contaminated soil samples.

The Death Rate of B. botulinus Spores at 100 C.

Chart 3 illustrates the death rate of the spores of B. botulinus suspended in a phosphate mixture and subjected to boiling water (99.5-100 C.). The survival of the spores has been determined in liquid mediums. Irrespective of the fact that the method furnishes only approximate data, it is evident that the mortality of the spores is a gradual process and follows in a general way the laws of logarithmic decline. The majority of spores are not exceptionally heat resistant. A relatively small number in this suspension survives the temperature of 100 C. for more than 90 minutes. Germination of these resistant spores may be delayed for several weeks or months. Uneven distribution of the resistant spores may favor the occurrence of so-called "skip-stops" (see paragraph The Influence of Concentration of Spores).

TABLE 3

Results of Time-Temperature Experiments

Strain	Grown in the Following Medium	Million Spores per 1 C.c.	P_H*	Heated in	Time in Minutes at 120 C.		115 C.		110 C.		105 C.		100 C.	
					+	—	+	—	+	—	+	—	+	—
62	Pea-peptic-digest broth	1,300	7.78	Pea-peptic digest 7.78 *	1	2	3	4	6	7	33	34	120	130
90	Pea-peptic-digest broth	1,800	8.01	Pea-peptic digest 8.01 *	1	2	3	4	9	10	44	48	160	170
47	Pea-peptic-digest broth	1,600	7.60	PO_4—6.85	1	2	6	7	15	16	65	70	285	300
97	Double strength veal infusion-peptic-digest gelatine	19,200	7.56	PO_4—6.88	4	5	9	10	28	30	90	100	315	330

* Final reaction of medium at the end of growth (10 days at 35 C.).
+ = survival time; — = destruction time.

The Time-Temperature Relationship

The time necessary to destroy a known suspension of spores in a medium of known hydrogen-ion concentration decreases as the temperature increases. This fact has been established on 7 different spore suspensions. The spores employed in the experiments have been grown either in pea-peptic-digest broth, or in double strength veal infusion-peptic digest gelatine; the suspensions have been heated in the same medium in which the spores have been produced or in phosphate solutions. The results of 4 series are shown in table 3 and chart 4. The curves in chart 4 show the destruction times at the different temperatures.

The Influence of Age of the Spores on the Thermal Death Rate

Four series of experiments have been made to determine the influence of age on the heat resistance of spores held at 35 C., 28 C. and in the icebox (average, 5 C.). In series 1, spore suspensions produced in pea juice-peptic digest broth have been tested after 4, 8, 12, 16, 20 and 30 days' incubation at 35 and 28 C. The spores of a culture kept at 35 C. for 30 days are less resistant than those incubated for only 4 days. This difference is, however, less marked for spores kept at 28 C. Spore production is slower at 28 C. The maximum resistance is reached on the 8th day and remains fairly consistent when kept at 28 C. The deterioration of the resistance is not influenced by the number of spores. In this series the average count remained practically constant irrespective of the low resistance of the old spores kept at 35 C.

In series 2, the influence of age has been determined by growing strain 90 in pea-peptic digest broth, with and without gelatine, and in neutral brain medium at 37 C., and 28 C. Resistance tests have been made in from 1½ to 186 days. The maximum resistances have been obtained in 1½ to 3½ days in pea gelatine, 2½ days in pea-peptic digest and 6 to 10 days in brain medium. at 37 C. After these periods deterioration of the resistance is rapid, although the number of spores present in the medium remains fairly constant. At 28 C. the maximum resistance in pea gelatine has been reached in 3½ days, and in peptic digest broth and brain medium in 8 days. After 15 to 20 days the resistance has dropped rapidly in all cultures irrespective of the number of spores per cubic centimeter of medium.

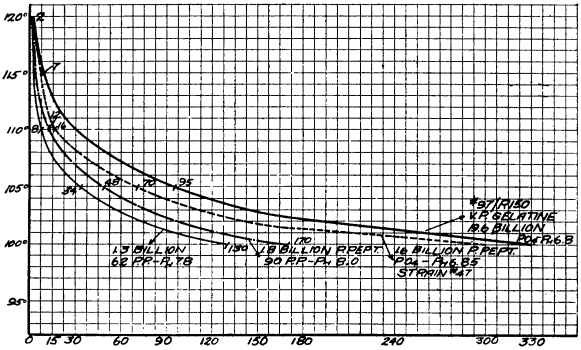

Chart 4.—Destruction curves.

The third series determined, in duplicate sets, the maximum resistance of spores produced in double strength veal-infusion-peptic digest-10%-gelatine broth at 37 C. Samples have been taken from the same flasks on the 2nd, 4th, 6th and 10th day. Abundant spores have been noted from the 4th day on, and the resistance has remained constant when tested on the 6th and 10th day.

The fourth series confirmed the previous observations. Furthermore, it demonstrated that cultures or spore suspensions kept in the icebox (5 C.) retain their maximum resistance for at least 30 days. Deterioration is, however, noted after a storage of 60 days. Cultures in double strength veal infusion-peptic digest-10%-gelatine broth containing excellent spores exhibit a gradual decrease in resistance after 21 days when kept at 37 C. This deterioration increases on incubation and is quite marked after 60 days. These experiments definitely indicate that the young spores, probably those of the first generation, are the most heat resistant. A well buffered medium, which furnishes abundant food material for progressive growth, produces, as a rule, spores of average or low resistance. Furthermore, any factor, as, for example, body temperature, which favors after the 10th day the continuous development of new spore-cycles, is conclusive to the production of abundant but poorly resistant spores. Spore suspensions in the moist state are preserved in the icebox for periods not exceeding 30 days.

THE RESISTANCE OF DRIED SPORES

On account of the great variability in the heat resistance of moist spores produced under ideal conditions, it is advisable to employ dried spores for a comparative study of the various factors which are ultimately responsible for their death. Numerous experiments have been conducted to standardize the procedures of dehydration of moist spore suspensions. In brief, it has been found that dried spores are somewhat less resistant than moist spores. Some of the factors responsible for this difference are in the course of investigation. The methods employed to prepare dried spores are briefly as follows: Washed spore suspensions are mixed with sterile sand. The moist material is smeared in Petri dishes and dried in vacuo over H_2SO_4 $CaCl_2$ or P_2O_5. Rapid drying in from 4 to 6 hours in vacuo at 34 C. (93 F) has produced the best results, and spores with a fairly high degree of heat resistance have been secured. Over a period of 347 days the resistance has remained constant and it appears that perfectly dry spores can be preserved equally well at 37 C., room temperature (20 C.), or in the icebox. Dried spores were found to be excellently suited for comparative studies, and their use can be highly recommended.

TABLE 4

RESULTS OF TESTS MADE TO DETERMINE RELATIONSHIP OF CONCENTRATION OF SPORES TO HEAT RESISTANCE

Strain	Number of Spores per 1 C c	Resistance in Min. at 105 C.		Resistance in Min. at 100 C.	
		+	—	+	—
90	900,000,000	44	48	...	...
	9,000.000	34	36	...	...
	90.000	18	20	...	...
	900	12.	14	...	...
	9	..	2	...	...
90	628,000,000	33	36	...	...
	6,280,000	33	36	...	...
	62,800	24	27	...	...
	6,280	15	18	...	...
	628	10	15	...	...
	63	..	5	...	...
97	72,000,000.000	..	..	230	240
	1,640,000,000	..	..	120	125
	32,800.000	..	..	105	110
	650,000	..	..	80	85
	16.400	..	..	45	50
	328	..	..	35	40

+ = survival time; — = destruction time.

THE INFLUENCE OF CONCENTRATION OF SPORES

The relationship of the concentration of the spores to the heat resistance has been determined on 15 different spore suspensions. The results secured in 3 tests are shown in table 4.

From the foregoing data it is evident that the greater the concentration, the greater the resistance. On account of the remarkable variability of the resistance of B. botulinus spores produced of one and the same strain under identical conditions, this condition holds true only for the dilutions of a given suspension. It is a common experience to encounter cultures with less than one million spores, although their resistance surpasses that of others containing several billion organisms per c c.

In this connection, attention is again called to the phenomenon of retarded germination and the occurrence of so-called "skip-stops" within the first 90 days of incubation. Subcultures prepared from heated suspensions have germinated after an incubation of 378 days at 36-37 C. The cultures produced a virulent toxin and presented typical morphologic and biochemical characteristics.

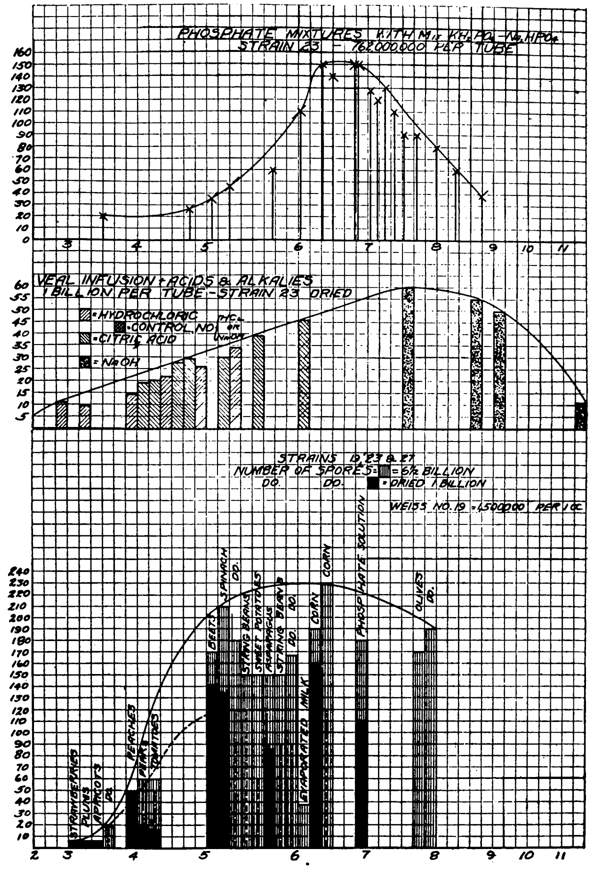

Chart 5.—Resistance of B. botulinus spores in phosphate mixtures, veal infusion of varying PH values by the addition of acid or alkali and food juices.

It has been frequently observed that subcultures made from suspensions which have been heated for long periods presented growth sooner than those heated for shorter times. These so-called "skip-stops" are believed to be the result of an uneven distribution of a relatively small number of very heat resistant spores. The occurrence of these "skip-stops" can, in part, be reduced by the preparation of carefully strained, uniform suspensions and by prolonged incubation. Subcultures prepared from diluted unheated or slightly heated spore suspensions (80 degrees for 1 hour or 100 degrees for 2 minutes) exhibit the phenomenon of retarded germination and "skip-stops." These and similar observations obviously indicate that the germination of a few spores of B. botulinus and B. sporogenes in suitable culture mediums is irregular and sometimes retarded for considerable periods.

The Effect of the P_H on the Thermal Death Rate

The effect of the hydrogen and hydroxyl-ion concentration on the heat resistance of spores of B. botulinus has been determined by suspending carefully washed spores in 4 different series of solutions of varying P_H values: (a) phosphate mixture series (M/15 Na_2HPO_4 and KH_2PO_4) varying from P_H 3.50 to 8.80; (b) 2% Difco peptone solution; (c) double strength veal infusion, and (d) spinach juice (extract of fresh leaves in boiling tap water for 1 hour) with varying amounts of N/10 acid or alkali.

Chart 5 illustrates the heat resistance of spores in phosphate mixtures. It is evident that the maximum resistance is on the acid side (about P_H 6.3 to 6.9. Final conclusions cannot be drawn from this series since prolonged incubation may change the available data.

TABLE 5

Effect of Heat on Spores of B. botulinus Suspended in Acidified Spinach Juice

Hydrochloric Acid				Citric Acid			
P_H	Resistance at 100 C. in Minutes		Retarded Germination, Days	P_H	Resistance at 100 C. in Minutes		Retarded Germination, Days
	+	—			+	—	
Control 5.05	45	50	7	Control 5.26	60	65	10
4.80	45	50	10	4.92	45	50	29
4.50	40	45	8	4.77	40	45	10
4.38	35	40	58	4.69	35	40	9
4.31	24	26	20	4.54	30	35	7
4.16	20	22	8	4.50	30	35	29
4.11	15	20	7	4.37	25	30	8
3.98	10	15	4	4.34	20	25	9
3.81	10	15	5	4.31	20	25	9
3.70	10	15	10	4.31	18	20	9

+ = survival time; — = destruction time.

The effect of 0.003 to 0.05 normal hydrochloric and citric acid and of 0.015 to 0.074 normal sodium hydroxide in double strength veal infusion on the spores is also shown in chart 5. The influence of the hydrogen-ion concentration on the heat resistance is marked below P_H 5.0 and above P_H 10.0 Malic, acetic, succinic and tartaric acid produce in a general way the same effect; below P_H 5.0 the resistance is greatly decreased. Hydrochloric and acetic acids are distinctly more toxic, while succinic, citric, malic and tartaric act in the order named. The behavior of the spores in acid or alkaline peptone solutions is similar to that in veal infusion.

The effect of heat on the spores of B. botulinus suspended in acidified spinach juice is shown in table 5. Definite amounts of hydrochloric and citric acids varying from 0.00025 to 0.027 normal were added to the spinach juice obtained as stated to produce a series of P_H values varying from 5.05 to 3.70 for hydrochloric acid and of P_H 5.26 to 4.31 for citric acid. This juice is rich in buffer substances and remained unaltered until 0.003 normal acid was added.

In the P_H range 4.5 to 5.5 little or no effect of the acid is noted; below 4.5 the decrease in the resistance is marked, as shown in table 5. It has been impossible to test the effect of alkalies on account of the profound chemical changes produced in the spinach juice by the addition of sodium carbonate or hydroxide.

THE EFFECT OF SODIUM CHLORIDE AND POTASSIUM CHLORIDE ON THE THERMAL DEATH RATE

Dried spores of several strains have been exposed to varying concentrations of sodium chloride in double strength veal infusion at different temperatures. No reduction in the heat resistance has been noted until the concentration has reached 8% sodium chloride. Ten and 20% solutions of sodium chloride decrease the resistance, while, on the other hand, the addition of 0.5 and 1% sodium chloride makes the destruction more difficult than in a 2 or 3% solution. These observations are briefly summarized in table 6.

An extensive series of tests has been carried out to determine the effect of holding vegetative forms and spores in 20% sodium and potassium chloride solutions with and without double strength veal infusion and Difco peptone (2%). It is shown that the Na-ion is more toxic than the K-ion, since the resistance is conclusively much less in sodium than in potassium solutions.

TABLE 6

EFFECT OF SALT ON THERMAL DEATH RATE

Spores Suspended in	P_H	Resistance at 100 C. in Minutes	
		+	—
Veal infusion— 0.0 NaCl	6.03	35	40
Veal infusion— 0.5% NaCl	5.94	45	50
Veal infusion— 1.0% NaCl	5.94	50	55
Veal infusion— 2.0% NaCl	5.83	30	35
Veal infusion— 4.0% NaCl	5.75	30	35
Veal infusion— 5.0% NaCl	5.70	35	40
Veal infusion— 6.0% NaCl	5.65	30	35
Veal infusion— 8.0% NaCl	5.51	25	30
Veal infusion—10.0% NaCl	5.50	20	24
Veal infusion—20.0% NaCl	5.06	10	12
M/15 phosphate mixture	6.92	105	110

+ = survival time; — = destruction time.

Spore suspensions held in 20% salt solutions become more resistant to heat during an incubation of 60 days at 37 C. Spores of considerable resistance are present even after an incubation of 340 days at this temperature.

Spores heated in peptone solutions without salts or meat extractives exhibit a relatively low heat resistance, as is well illustrated by the data in table 7.

The influence of certain amino groups, polypeptides, on the heat resistance of spores is suggested by these observations and deserves for numerous reasons careful investigation.

THE RESISTANCE OF B. BOTULINUS SPORES IN FOOD JUICES

The heat resistance of 4 different spore suspensions of B. botulinus has been determined in the following juices of 17 varieties of canned food: corn, spinach, asparagus, string beans, peas, ripe olives, evaporated milk, beets, sweet potato, tomato, sauerkraut, pears, peaches, apricots, plums, strawberries and cherries. The spores have been washed twice in sterile tap water, resuspended in tap water, and 5 c c of the suspension has been added to 45 c c of the food juice.

In chart 5 the results recorded with 2 different spore suspensions are shown in graphic form. It will be noted that above P_H 5.0 some factor other than hydrogen-ion concentration has a profound influence on the time necessary for the sterilization of foods. In this respect the results differ fundamentally from those previously determined on phosphate mixtures or veal infusion acid and alkali solutions, as shown in chart 5. For example, it is striking that spores heated in ripe olives with a P_H 7.93, corn P_H 6.35 and spinach P_H 5.05 have approximately the same resistance, while, on the other hand, in asparagus with a P_H 5.25 and 5.55 sterilization is more readily accomplished. In food juices with a P_H value below 4.5 the hydrogen-ion concentration has a marked influence on the destruction of B. botulinus spores. Careful consideration should be given to these facts in estimating the processing times and temperatures applicable to the practical canning of food.

TABLE 7

HEAT RESISTANCE OF SPORES IN VARIOUS SOLUTIONS

Spores Suspended in	P_H	Resistance at 100 C. in Minutes	
		+	—
Phosphate solution	6.92	110	120
Peptic digest broth	7.00	120	130
Double strength veal infusion	6.70	90	100
2% aminoids	5.65	60	65
	5.65	60	65
2% Difco peptone	7.40	40	50
	7.08	30	35
2% Witte's peptone	7.45	20	25
	7.45	20	25

SUMMARY

The heat resistance of 109 strains of B. botulinus (78 type A, 30 type B and one nontoxic) from various sources varies from three to 80 minutes at 105 C. The spores were produced in pea peptic digest broth, P_H 8.0, and heated in a phosphate solution P_H 7.00-7.12.

The heat resistance of 33 strains of B. sporogenes varies from 10 to 150 minutes at 100 C.; four to 45 minutes at 105 C. and one to 12 at 110 C.

The heat resistance of 24 strains of B. tetani varies from 15 to 90 minutes at 100 C. and three to 25 minutes at 105 C.

Strains of B. bifermentans, B. centrosporogenes, B. histolyticus, B. oedematiens, B. aerofoetidus and the anaerobe isolated by Bengston are killed within six minutes at 105 C. One strain of B. welchii survived 24 minutes at 105 C. but was destroyed in 27 minutes.

The maximum heat resistance of B. botulinus spores artificially produced under the most favorable conditions for growth and heated in a phosphate solution of a P_H 7.0 is as follows:

4 minutes at 120 C. (248 F.)
10 minutes at 115 C. (239 F.)
32 minutes at 110 C. (230 F.)
100 minutes at 105 C. (221 F.)
330 minutes at 100 C. (212 F.)

These results represent actual survival times at the given temperatures.

The heat resistance of spores of the same strain of B. botulinus varies considerably, depending on several factors, some of which are unknown.

The average resistance of the spores of B. botulinus, as found in approximately 20 grams, either of naturally or artificially contaminated soil is less than three hours at 100 C. (212 F.).

The death rate of B. botulinus spores is gradual and follows in a general way the laws of logarithmic decline. The majority of the spores are not exceptionally heat resistant.

Young moist spores, probably those of the first generation, appear to be the most heat resistant. Spore suspensions in the moist state are best preserved in the icebox for periods not exceeding 30 days. Prolonged incubation of spores held at 20 C. or 35 C. tends to lessen the resistance.

The heat resistance of dried spores has remained constant over a period of 347 days, and it appears that perfectly dry spores can be preserved equally well at 37 or, 20 C. or in the icebox.

It is evident that the heat resistance increases as the concentration increases determined by dilutions of a given suspension. However, cultures with less than one-million spores may have a greater heat resistance than others containing several billion organisms per c c.

Subcultures from heated spore suspensions of B. botulinus have germinated after an incubation of 378 days at 36-37 C.

The hydrogen ion concentration of different solutions affects the thermal resistance of B. botulinus spores.

Spores heated in concentrations of sodium chloride from 0.5% to 6% do not decrease in heat resistance. The presence of 8, 10 and 20% solutions greatly decreases the resistance while 0.5% and 1.0% appear to enhance it. Spores heated in peptone solutions without salts or meat extractives exhibit a relatively low heat resistance.

The heat resistance of B. botulinus spores in the juices of 17 varieties of canned food shows a variation from less than 10 minutes to 230 minutes at 100 C.